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L.S.WICKER, G.GUELDE, I.SCHER AND J.J.KENNY

J. Vorosmarti, CAPT, MC, USN

Commanding Officer

Naval Medical Research Institute

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ANTIBODIES FROM THE Lyb-5⁻ B CELL SUBSET PREDOMINATE IN THE SECONDARY IgG RESPONSE TO PHOSPHOCHOLINE[1]

LINDA S. WICKER,[2] GRETCHEN GUELDE, IRWIN SCHER, AND JAMES J. KENNY[3]

From the Naval Medical Research Institute and the Departments of Microbiology and Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD

The X-linked CBA/N immune defect was used to investigate the role of the Lyb-5⁻ B cell subset in phosphocholine- (PC) specific memory responses. Immune-defective mice, which express only the Lyb-5⁻ B cell subset, are unable to mount a primary or secondary T15⁺, IgM response to PC but can produce a substantial secondary IgG response. The majority of these IgG anti-PC antibodies are T15⁻ and can be inhibited by phenylphosphocholine, but not PC. Normal mice, which possess Lyb-5⁺ and Lyb-5⁻ B cells, produce both IgM and IgG anti-PC antibodies; however, there is a striking difference in the idiotype and fine specificity of antibodies expressed by these two classes. The IgM anti-PC antibodies are T15⁺ and PC-inhibitable, whereas the IgG antibodies are identical to those observed in the immune-defective mice, i.e., T15⁻ and PC-noninhibitable. This unexpected difference in both idiotype and fine specificity between IgM and IgG anti-PC antibodies results from activation of different B cell subsets. Lyb-5⁺ B cells produce T15⁺, PC-inhibitable IgM antibodies, whereas T15⁻, PC-noninhibitable IgG antibodies are produced by Lyb-5⁻ B cells. These data indicate that a majority of the thymus-dependent, anti-PC IgG memory response arises from Lyb-5⁻ B cells.

The immune response to phosphocholine (PC)[4] on T-independent (TI) and T-dependent (TD) carriers has been studied extensively (1, 2). A striking finding has been the appearance of a predominant idiotype, T15, in the primary and secondary IgM anti-PC response (3–5). In mouse strains such as BALB/c, greater than 90% of primary and secondary IgM plaque-forming cells (PFC) bear the T15 idiotype. A large amount of IgG anti-PC antibody is also produced in the secondary response to PC on TD carriers (6, 7). Claflin and Cubberley (7) have shown that anti-PC antibodies bearing the T15 idiotype are present in all the IgG subclasses; however, Chang *et al.* (8) have recently suggested that a second group of anti-PC antibodies (group II) represents the major portion of the IgG1 and IgG2 isotypes. This second type of antibody is T15⁻ and is not inhibitable by free PC, but is inhibited with *p*-nitrophenylphosphocholine (NPPC). Thus, these anti-PC antibodies recognize PC only in conjunction with the phenyl ring that links it to the carrier.

Recent studies from this laboratory (9) and others (10, 11) have demonstrated that mice expressing the X-linked CBA/N *xid* gene fail to make a significant IgM anti-PC response, and that their substantial IgG secondary anti-PC response is predominantly T15⁻. Because CBA/N mice lack Lyb-5⁺ B cells (12), we postulated that the IgM, T15⁺, anti-PC antibodies of normal mice are derived from Lyb-5⁺ B cells, whereas their IgG, T15⁻, anti-PC antibodies are derived from Lyb-5⁻ B cells.

In this study we compare the anti-PC memory expression of B cells from mice that express *xid*, and thus have only Lyb-5⁻ B cells, to the response of B cells from normal mice, which have both Lyb-5⁺ and Lyb-5⁻ B cells. These data indicate that Lyb-5⁻, PC-specific B cells predominate in the secondary anti-PC response and that the antibodies produced by these cells are mainly T15⁻, inhibitable with *p*-aminophenylphosphocholine (APPC) but not PC.

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[2] L. S. Wicker is a National Research Council Resident Research Associate of the Naval Medical Research and Development Command.

[3] Reprint requests should be addressed to James J. Kenny, Department of Microbiology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814.

[4] Abbreviations used in this paper: TI, thymus independent; TD, thymus dependent; PC, phosphocholine; NPPC, *p*-nitrophenylphosphocholine; APPC, *p*-aminophenylphosphocholine; CB, (CBA/N × BALB/c)F_1 progeny; CD, (CBA/N × DBA/2)F_1 progeny; DPPC, *p*-diazophenylphosphocholine; ARS-BSA, bovine serum albumin conjugated with *p*-diazophenylarsonate; PC-BSA, bovine serum albumin conjugated with DPPC; V_H-4, heavy chain found in most PC-binding antibodies; V_HPC, common idiotype associated with the V_H-4 heavy chain; KLH, keyhole limpet hemocyanin; Hy, *Limulus polyphemus* hemocyanin, PC-Hy, Hy conjugated with DPPC.

MATERIALS AND METHODS

Animals and antigens. (CBA/N × BALB/c)F_1 (CB) and (CBA/N × DBA/2)F_1 (CD) male and female mice were obtained from Dominion Laboratories (Dublin, VA). Because *xid* is on the X chromosome and is expressed in only the hemizygous or homozygous form, F_1 male mice from these crosses are immune defective, whereas their F_1 female littermates are normal. Keyhole limpet hemocyanin (KLH) and *Limulus polyphemus* hemocyanin (Hy) were conjugated to PC using *p*-diazophenylphosphocholine (DPPC) as described by Chesebro and Metzger (13). The APPC precursor of DPPC was obtained from Biosearch (San Rafael, CA).

Serum analysis. Analysis of sera for the T15 and V_HPC (see Abbreviations) idiotypes and for total anti-PC IgG and IgM levels was performed as previously described (9).

Preparation of spleen cells for adoptive transfer. CB male and female mice were primed with either 200 μg KLH or 100 μg Hy conjugated with DPPC (PC-Hy) in CFA 6 wk before use. PC-Hy-primed spleen cells were treated with monoclonal anti-Thy-1.2 (generously provided by Dr. Phil Lake) and rabbit complement (C). B cell preparations contained less than 5% Thy-1.2⁺ cells as determined by analysis on the fluorescence-activated cell sorter (FACS) using biotin-conjugated anti-Thy-1.2 and fluorescein-labeled avidin (obtained from Becton Dickinson, Sunnyvale, CA). KLH-primed T-helper cells were obtained after depletion of Ig⁺ cells on rabbit anti-mouse κ-coated petri dishes as described by Mage *et al.* (14). T cell preparations contained less than 5% Ig⁺ and/or Ia⁺ cells. 2 × 10^7 PC-Hy primed B cells and 10^7 KLH-primed T cells from either male or female mice were injected i.v. into irradiated (900 R) CB female recipients. Mice were then immunized

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i.p. with 50 μg of PC-KLH in saline. Serum was collected and PFC analysis was performed 7 days after cell transfer. Irradiated, unreconstituted, F_1 female recipients produced no PFC when immunized with antigen. Indistinguishable results were obtained in adoptive transfer assays when immune defective males were utilized as the irradiated recipients (data not shown).

PFC analysis. Anti-PC PFC were determined by the Cunningham and Szenberg (15) slide method. Sheep red cells were coupled with DPPC according to Claflin *et al.* (5). IgG PFC were developed in the presence of goat anti-mouse IgM (kindly provided by Dr. James Mond) and were facilitated with a combination of isotype-specific antisera.

RESULTS

The secondary in situ response to PC-KLH in normal and immune-defective mice. Normal female and immune-defective CD male mice were immunized twice with PC-KLH. Seven days after the second injection of antigen, the spleen cells of individual mice were analyzed for total IgM and IgG anti-PC PFC and for the number of PFC inhibitable with either anti-T15 idiotypic antibodies, 10^{-3} M PC, 10^{-3} M APPC, 10^{-5} M bovine serum albumin conjugated with DPPC (PC-BSA), or 10^{-5} M BSA conjugated with *p*-diazophenylarsonate (ARS-BSA). The data in Table I show that normal CD female mice produced both IgM and IgG anti-PC antibodies; however, the specificity and idiotype of the IgM and IgG PFC were quite different. The IgM anti-PC antibodies were predominantly T15⁺ and inhibitable by free hapten, whereas the IgG PFC were mostly T15⁻ and PC-noninhibitable. Although greater than 95% of the IgG PFC could be inhibited by PC-BSA or APPC, none of these PFC were inhibitable with ARS-BSA, a compound containing the same diazophenyltyrosine linkage as PC-BSA. These findings suggest that the IgG PFC are PC specific but also recognize the aminophenyl group as part of the total epitope.

Immune-defective mice failed to produce secondary IgM anti-PC PFC *in situ*, but produced as many secondary IgG PFC as normal females (Table I). The IgG PFC from immune-defective males were similar to the IgG PFC from normal females in that greater than 90% of these PFC were T15⁻ and PC-noninhibitable.

These data strongly suggest that the majority of the IgG, T15⁻, secondary PFC in both normal female and defective male CD mice arise from the Lyb-5⁻ B cell subset, and that these antibodies recognize PC in the context of the aminophenyl ring. In contrast, the secondary, IgM, T15 dominant, PC-inhibitable, anti-PC response appears to come from Lyb-5⁺ B cells, which are present in CD female but not male mice. Similar results were obtained in the anti-PC-KLH responses of CB male and female mice (data not shown).

Adoptive transfer analysis of secondary anti-PC antibodies. Bottomly and Mosier (16) have suggested that a T15 idiotype-specific helper cell is absent in mice expressing the *xid* gene, and that these cells are necessary for maximal expression of T15⁺-anti-PC precursors. To assess the role of T helper cells in regulating the T15⁺-IgM and T15⁻-IgG phenotypes described above, adoptive transfers were performed in which KLH-primed T cells and PC-primed B cells from defective males and normal females were mixed and analyzed for their anti-PC response (Table II). Primed T and B cells from CB male or female mice were transferred to irradiated (900 R) female recipients, which were then immunized with 50 μg of PC-KLH in saline. Spleen cells were harvested and assayed for anti-PC PFC 7 days later.

When PC-primed female B cells were analyzed with either male or female T helper cells, large numbers of T15⁺, PC-inhibitable, IgM PFC were seen. The IgG anti-PC response obtained from female B cells with *either male or female helper* T cells was low in T15 idiotype and only 50% PC-inhibitable. Thus, in both the IgM and IgG responses, it is the B cell and not the source of T helper cells that determines the character of the anti-PC antibody.

PC-primed B cells from immune defective male mice transferred with either male or female T helper cells gave rise mainly to IgG PFC that were T15⁻ and PC-noninhibitable. A significant IgM anti-PC PFC response was observed in mice that received male B cells and T helper cells from males or females. These IgM PFC displayed the same phenotype as most of the female and male IgG PFC in that they were PC-noninhibitable and T15⁻. As noted above, the source of the B cell, not the T helper cell, determined the quality of antibody produced.

The serum anti-PC antibodies obtained from the adoptively transferred mice were analyzed for the expression of the T15 and V_HPC idiotypes and for inhibition of binding by PC and APPC (Table III). The V_HPC idiotype is the public idiotype held in common by a large number of PC-binding myelomas and hybridomas that share the same V_H-4 sequence (17–20). Male

TABLE I

The secondary in situ response to PC-KLH by normal and immune-defective (CBA/N × DBA/2)F_1 mice[a]

Spleen Cell Source	No. of Ig PFC/10^6 Spleen Cells	% T15⁺ [b]	% PC-Inhibitable[b]	% APPC-Inhibitable[b]	% PC-BSA-Inhibitable[b]	% ARS-BSA-Inhibitable[b]
	IgM					
♀	61 (1.12)[c]	86	94	98	100	0
♂	3 (1.57)	n.t.[d]	n.t.	n.t.	n.t.	n.t.
	IgG					
♀	37 (1.37)	8	11	97	100	0
♂	40 (1.06)	2	9	98	100	0

[a] Mice were immunized i.p. with 200 μg PC-KLH in CFA. Spleen cells were assayed 7 days after boosting with 50 μg PC-KLH in saline.

[b] The percentage of T15⁺ PFC was determined by incorporating affinity-purified rabbit anti-T15 into the plaquing mixture at a final concentration of 1:1000. The percentage of PC-inhibitable, APPC-inhibitable, PC-BSA-inhibitable, and ARS-BSA-inhibitable PFC was determined by incorporating PC or APPC at 10^{-3} M and PC-BSA or ARS-BSA at 10^{-5} M in the plaquing mixture. Inhibition data are presented as the arithmetic mean of five individual mice. Standard errors of the mean were all less than 5%.

[c] Data are presented as the geometric mean (standard error of the mean) of five individual mice.

[d] n.t., not tested.

TABLE II

The B cell subset determines the idiotype and specificity of anti-PC antibodies

KLH-Primed CB T Cells Transferred[a] (10 × 10^6)	PC-Hy-Primed CB B Cells Transferred[a] (20 × 10^6)	B Cell Subset Present	IgM PFC/Spleen (× 10^{-3})	% T15⁺ [b]	% PC-Inhibitable[b]	IgG PFC/Spleen (× 10^{-3})	% T15⁺	% PC-Inhibitable
♀	♀	Lyb-5⁺ + Lyb-5⁻	720 (1.14)[c]	90	99	1,150 (1.16)	27	51
♂	♀	Lyb-5⁺ + Lyb-5⁻	456 (1.04)	90	98	684 (1.22)	7	53
♂	♂	Lyb-5⁻	12 (1.52)	4	14	185 (1.39)	1	4
♀	♂	Lyb-5⁻	17 (1.19)	8	2	289 (1.18)	8	2

[a] B and T cells transferred individually gave less than 5% of the experimental values. CB T and CB B cells were injected i.v. into CB female recipients irradiated with 900 rad 4 hr before the transfer. Recipients were challenged with 50 μg PC-KLH i.p.

[b] Please refer to Table I.

[c] Data are presented as the geometric mean (standard error of the mean) of five individual mice.

TABLE III

Serum analysis of anti-PC-KLH adoptive transfer[a]

Cells Transferred		µg Anti-PC Antibody/ml Serum							
				IgM[c]			IgG[c]		
T cells	B cells	T15 Idiotype[b]	V_HPC Idiotype[b]	anti-PC	in 10^{-3} M PC	in 10^{-3} M APPC	anti-PC	in 10^{-3} M PC	in 10^{-3} M APPC
♀	♀	725	1419	1450	54	49	1950	1500	52
♂	♀	482	1294	1150	51	42	2450	680	32
♂	♂	<2.5	156	29	31	20	300	235	23
♀	♂	<2.5	226	46	40	30	490	320	26

[a] This table represents the serum analysis of the experiment detailed in Table II. CB donors and recipients were utilized in the transfer.

[b] T15 and V_HPC idiotype-positive antibodies were evaluated as described in Reference (9).

[c] *Sera were diluted into PBS in the presence or absence of* 10^{-3} M hapten, and each dilution was then added to two separate wells of a polyvinyl chloride plate coated with PC-BSA. After incubation and washing, class-specific rabbit anti-mouse IgM and IgG antibodies were added, followed by ^{125}I-labeled guinea pig anti-rabbit Ig. Standard curves were generated using T15⁻ IgM and IgG hybridoma antibodies obtained from Dr. J. L. Claflin.

B cells produced little T15⁺ serum antibody but produced significant levels of V_HPC positive antibodies (Table III). In contrast, female B cells produced high levels of both the T15 and V_HPC idiotypes. However, V_HPC-bearing antibodies did not appear to make up the entire anti-PC response in either male or female B cells. A comparison of the total IgM and IgG anti-PC responses (Table III) with the V_HPC values shows that approximately 50% of the anti-PC response is V_HPC positive. This has been recently confirmed at the PFC level using anti-V_HPC serum to inhibit anti-PC PFC (unpublished data). Data in Table III also show that 10^{-3} M PC inhibited greater than 90% of the female IgM and a substantial portion of the IgG anti-PC antibodies produced in mice reconstituted with female B cells. In contrast, free PC had little inhibitory effect on the IgM or IgG antibodies produced in mice reconstituted with male B cells. Greater than 90% of the IgG anti-PC-KLH response from male and female B cells was inhibited with 10^{-3} M APPC. However, the small IgM response observed with male B cells was only 30 to 50% APPC-inhibitable.

DISCUSSION

The data presented in this publication suggest that the anti-PC response in normal mice is composed of two major antibody phenotypes and that these antibodies arise from different B cell subsets. The Lyb-5⁺ B cell subset, which is absent in immune-defective male mice, produces IgM, T15⁺, PC-inhibitable antibodies. The Lyb-5⁻ subset, which is present in both normal female and immune-defective male mice, produces mainly IgG, T15⁻ antibodies that are inhibitable with APPC but not free PC. This latter phenotype predominates in the secondary response of normal female mice and comprises almost the total response in immune-defective male mice. These data support the previous observation of Chang and Rittenberg (21) that there is a striking difference between the BALB/c anti-PC IgM and IgG memory responses. They observed that the IgM response was PC-inhibitable and greater than 90% T15, whereas the IgG response was PC-noninhibitable and low in T15 idiotype. The results of our study indicate that this shift in both idiotype and fine specificity can be explained by the preferential expression of memory IgG PFC from Lyb-5⁻ B cells. The majority of antibody produced by this subset, as measured in CD and CB defective mice, is T15⁻ and PC-noninhibitable.

The results obtained from adoptive transfer of male and female primed B and T cells demonstrate that the antibody quality as measured by idiotype and fine specificity is determined by the B cell subset, not the helper T cell source (Table II). Female B cells produced IgM, T15⁺, PC-inhibitable responses with help provided by either male or female T cells. These results contrast with those of Bottomly and Mosier (16): however, studies from this laboratory (Kenny *et al.*, manuscript submitted for publication) and from Quintans *et al.* (22) have failed to reveal a difference between the T cell help obtained from defective male and normal female mice in the anti-PC response. In addition, male B cells were unable to produce IgM, PC-inhibitable anti-PC antibodies even when female helper T cells were provided. Although male B cells produced fewer PFC than female B cells, and male T cells had a lower specific T-helper activity than female T cells, these quantitative differences did not influence the relative idiotype expression or fine specificity of the anti-PC antibodies produced by male or female B cells. This might be explained by the fact that a lower absolute number of male T and B cells was transferred. Male cell preparations contained twice as many null cells as female spleen cells, and no attempt was made to normalize the male and female cell preparations to equivalent numbers of Ig⁺ or Thy-1.2⁺ cells before cell transfer. Also of interest was the large increase in anti-PC PFC when the adoptive transfer responses were compared with the *in situ* secondary responses (Table II *vs* Table I). The mechanisms responsible for the 100- to 200-fold higher response in adoptive transfer are under investigation.

The majority of antibodies from the Lyb-5⁻ subset appear to be specific for the PC hapten in association with the amino-phenyl ring, because they are inhibited by APPC and PC-BSA but not by free PC or ARS-BSA. The antibodies produced by the Lyb-5⁻ subset appear to be identical to group II anti-PC antibodies produced by normal mice described previously by Chang *et al.* (8). Chang *et al.* (8) have reported that BALB/c NPPC-inhibitable IgG antibodies (group II) cannot be inhibited with *p*-nitrophenylphosphate. Therefore, the entire PC moiety is important for binding by these NPPC-specific antibodies. Further evidence for the PC specificity of the male-derived antibodies comes from the observation that a portion of these PC-noninhibitable antibodies are V_HPC positive and therefore express the V_H-4 heavy chain, which is present in the PC-binding myeloma and hybridoma antibodies described by Claflin *et al.* (20) and Gearhart *et al.* (18). This anti-PC clonotype (V_HPC positive, PC-noninhibitable) has also been observed in the analysis of secondary precursor cells from defective males in the splenic focus assay (Dr. E. Metcalf, personal communication). The appearance of IgG anti-PC antibodies that are V_HPC negative indicates that both male and female mice use a heavy chain distinct from V_H-4 for binding PC or APPC. Indeed, some PC-binding hybridomas obtained from anti-T15 suppressed BALB/c mice do not have the V_H-4 heavy chain and instead express V_H-12 (23). A striking difference observed between the secondary *in situ* anti-PC-KLH response and the secondary adoptive transfer response was the percentage of female IgG PFC that were PC-inhibitable. *In situ* female re-

sponses were only slightly PC-inhibitable and were indistinguishable from the male response. On the other hand, the adoptive transfer IgG anti-PC response of female B cells was 50% PC-inhibitable, whereas the male B cell response remained PC-noninhibitable. Experiments are in progress to determine whether this T15$^-$, PC-inhibitable clonotype is derived from the Lyb-5$^+$ or Lyb-5$^-$ subset of female B cells. We are also performing adoptive transfer experiments with anti-Lyb-5 and C-treated CD female B cells to confirm our hypothesis that T15$^+$, IgM B cell precursors are restricted to the Lyb-5$^+$ B cell subset in normal mice and that the majority of IgG precursors arise from the Lyb-5$^-$ subset and are APPC specific.

What could possibly induce the apparent anti-PC repertoire divergence observed between Lyb-5$^+$ and Lyb-5$^-$ B cells? Because Lyb-5$^-$ B cells are more easily tolerized than the Lyb-5$^+$ cells (24), we would like to propose that these cells are tolerized to PC as it appears on environmental antigens. This tolerance would eliminate most T15$^+$ clones from the Lyb-5$^-$ subset as well as other clones that would bind bacterial froms of PC. However, when Lyb-5$^-$ cells encounter PC in the context of the diazophenyl ring, a form of PC that is not likely to be a common environmental epitope, the APPC-specific clones can be activated. The induction of tolerance would not necessarily eliminate all Lyb-5$^-$, PC-inhibitable clones, because some of these clones would be unreactive with bacterial PC-containing antigens.

In summary, Lyb-5$^-$ B cells appear to be responsible for a high percentage of the IgG anti-PC memory response. The PC specificity and idiotype of these antibodies differ from the classical PC-inhibitable myeloma and hybridoma antibodies described by Claflin *et al.* (20) and Gearhart *et al.* (18). We propose that these monoclonal anti-PC antibodies, all of which express the V_H-4 heavy chain, do not accurately reflect the anti-PC repertoire arising from the Lyb-5$^-$ subset, and that the V_H-4 coding sequence is not the only heavy chain utilized in the anti-PC response.

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